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Mitosin (a New Proliferation Marker) Correlates with Clinical Outcome in Node-negative Breast Cancer[1]

Gary M. Clark,[2] D. Craig Allred, Susan G. Hilsenbeck, Gary C. Chamness, C. Kent Osborne, Diane Jones, and Wen-Hwa Lee

Department of Medicine, Division of Medical Oncology [G. M. C., S. G. H., G. C. C., C. K. O.], Department of Pathology [D. C. A.], and Department of Molecular Medicine, Institute of Biotechnology [D. J., W.-H. L.], University of Texas Health Science Center, San Antonio, Texas 78284

ABSTRACT

Tumor proliferation rate is an important prognostic factor in breast cancer, and S-phase fraction (SPF), as measured by flow cytometry, is the most clinically validated of several methods for measuring it. However, flow cytometry is not well suited to evaluating the formalin-fixed, paraffin-embedded tumors that are routinely available or to the increasing number of small breast cancers. These and other limitations have motivated research into alternative methods for measuring proliferation, including immunohistochemistry (IHC) against cell cycle-related antigens, which are better suited for the evaluation of small archival tissue samples. Mitosin is a recently described 350 kD nuclear phosphoprotein that is expressed in the late G_1, S, G_2, and M phases of the cell cycle but not in G_0. Using a new monoclonal antibody (14C10), this pilot study evaluated mitosin expression by IHC in a series of 386 node-negative, formalin-fixed, archival breast cancers and correlated the results with several prognostic factors and clinical outcome (median follow-up, 78 months; range 3–214 months). The median and range of mitosin positive cells were 7% and 1–47%, respectively. There was a strong positive correlation between mitosin and SPF ($r = 0.57$; $P = 0.0001$), and there were significant negative correlations with estrogen receptor, progesterone receptor, and patient age. Mitosin was not related to overall survival in this pilot study. However, in a univariate cutpoint analysis of disease-free survival (DFS), patients with high levels of mitosin (>9% positive cells) had significantly worse DFS than did patients with lower levels (68% *versus* 84% at 5 years, respectively). In a multivariate analysis of DFS, large tumor size (>2 cm) and high mitosin were the only independently significant predictors of recurrence (relative risks = 2.47 and 1.72, respectively) in a model containing the additional factors estrogen receptor, progesterone receptor, patient age, and SPF. These preliminary results suggest that mitosin as assessed by IHC may be superior to SPF as a prognostic factor in node-negative breast cancer, but additional studies are necessary to validate these promising findings.

INTRODUCTION

Alterations in the control of cell proliferation are important in the development and progression of many types of human cancer, and the overall rate of proliferation is indirectly related to clinical outcome. There are several techniques for estimating or measuring proliferation, including light microscopic mitotic index, thymidine labeling index, bromodeoxyuridine uptake, and DNA flow cytometry. Each of these methods has strengths and weaknesses, but none are ideal for measuring proliferation in formalin-fixed, paraffin-embedded tissue, which has become the standard method of processing and storing clinical samples. IHC[3] against cell cycle-related antigens is a more recent method for measuring proliferation that is better suited for evaluating routine archival tissue, and antibodies have been developed to a few antigens, such as Ki67, which have been used in preliminary studies with varying success. The ultimate success of IHC will depend on how specific the antigens are for dividing cells and how sensitive the antibodies against them are in archival tissue. So far, none of them is optimal.

Mitosin is a recently identified 350 kD nuclear phosphoprotein that is involved in cell division and, therefore, may be a suitable target for evaluating proliferation by IHC. It was identified through its binding to purified retinoblastoma protein, and it is expressed in the late G_1, S, G_2, and M phases of the cell cycle but is absent in G_0 (1). It associates with the centromere, spindle, and midbody of the mitotic apparatus during M and completely degrades following cytokinesis (1). The carboxy terminus of the molecule has been shown to be essential for the localization of mitosin in the nucleus (2).

We have developed several monoclonal antibodies to mitosin that are suitable for IHC in archival tissue. This pilot study used one of these antibodies (14C10) to measure mitosin expression by IHC in 386 archival node-negative human breast cancers. The results were compared to SPF, as determined by flow cytometry, several other clinical/pathological features, and patient outcome.

MATERIALS AND METHODS

Antibody Production and Characteristics. Balb/c mice were immunized with bacterially expressed fusion protein GST 10Bgl (containing amino acids 1759–2093 of mitosin). Injections of 100 μg of protein were given s.c., the first in Complete Freund's Adjuvant, followed by three boosts in Incomplete Freund's Adjuvant at 4–6-week intervals. A final boost of GST 10Bgl in sterile PBS was given i.v. 3 days prior to the fusion.

Isolated immune splenocytes were fused with NS1 mouse myeloma cells using 50% polyethyleneglycol essentially as described by Oi and Herzenberg (3). Successful hybridomas were initially screened by ELISA against GST and GST 10Bgl. Several clones that only reacted to GST 10Bgl were expanded, single-cell cloned, and retested by ELISA to confirm specificity. Five clones (2G8, 13E7, 14C10, 16G6, and 18E1) with strong GST 10Bgl activity were then injected into the peritoneum of *Balb/c* mice to form ascites. IgG was purified from the ascites using protein G Sepharose, dialyzed against PBS, and stored long-term at −80°C.

In a previous study using normal mouse kidney CV1 cells synchronized by different drugs to obtain uniform populations at different phases of the cell cycle, mitosin was shown to be expressed in late G_1, S, G_2, and M but not in G_0 (1). The specificity of the mitosin antibody used in this study was confirmed by immunoprecipitation against whole-cell lysates from the normal human breast epithelial cell line HBL-100. To obtain sufficient quantity of cells expressing mitosin, they were first arrested and synchronized at the G_1-S boundary for 24 h with hydroxyurea and released for 4 h prior to harvesting. The lysates were precipitated with each of the five purified antibodies described above, the products were separated on a polyacrylamide gel, Western blotted, and probed with antibody 14C10. Each antibody showed single-band

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[2] To whom requests for reprints should be addressed, at Department of Medicine, Division of Medical Oncology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284-7884. Phone: (210) 567-4749; Fax: (210) 567-6687; E-mail: gary@oncology.uthscsa.edu.

[3] The abbreviations used are: IHC, immunohistochemistry; SPF, S-phase fraction; GST, glutathione *S*-transferase; ER, estrogen receptor; PgR, progesterone receptor; DFS, disease-free survival; OS, overall survival.

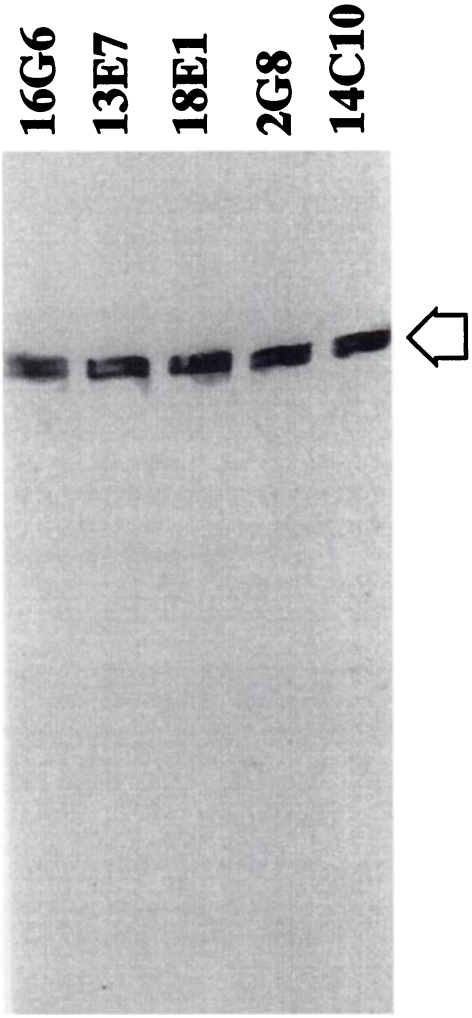

Fig. 1. Lysates from the normal human breast epithelial cell line HBL-100 were immunoprecipated with five separate monoclonal antibodies (16G6, 13E7, 18E1, 2G8, and 14C10) to mitosin, and a Western blot was probed with antibody 14C10. All antibodies showed specificity for a single product with a molecular weight consistent with mitosin.

specificity to a product with a molecular weight consistent with mitosin (Fig. 1). 14C10 was used in this study, based on its higher sensitivity in a test panel of archival breast cancers evaluated by IHC (data not shown).

Patients and Tumor Specimens. Tumors from 386 patients were included in this correlative study. All patients were diagnosed with node-negative breast cancer between 1973 and 1989, and their tumor specimens were obtained at the time of diagnosis prior to any systemic therapy. Median age at diagnosis was 60 years (range, 27–102 years). Seventy-four % of patients received no additional therapy. Adjuvant systemic therapy was administered to 26% of the patients (12% endocrine therapy alone, 9% chemotherapy alone, and 5% combined endocrine and chemotherapy). Patients were followed for disease recurrence and death, as described previously (4). Median follow-up was 78 months (3–214 months). At the time of analysis, 26.4% of the patients had experienced disease recurrence, and 22.5% had died.

Tumors were obtained fresh at the time of diagnostic biopsy or mastectomy, snap-frozen, and pulverized in liquid nitrogen for steroid receptor analyses and flow cytometry and stored at −70°C for 1–15 years. Permanent histologic sections for IHC were prepared from 50 mg of pulverized tumor, as described previously (5). Briefly, frozen tissue was thawed and rehydrated in PBS, fixed in 10% neutral-buffered formalin for 6–8 h, pelleted in a 2.5% agar matrix, and then routinely processed to paraffin blocks. The resulting 5-μm sections were composed of 10–20 histologically intact tissue fragments, measuring 0.1–1.0 mm each, and contained from 200 to 1000 total tumor cells.

Steroid Receptors. ER levels were measured by the dextran-coated charcoal method, as described previously (6). From 1973 to 1984, [^{3}H]estradiol was used as labeled ligand. During the same period, PgR levels were measured by sucrose density gradient (7). Since 1985, the standard multipoint dextran-coated assay has been modified to incorporate ^{125}I-labeled estradiol and [^{3}H]R5020 in a single assay, allowing for the simultaneous determination of both ER and PgR levels (8).

Flow Cytometry. Flow cytometry was carried out as described previously (8, 9). Briefly, approximately 100 mg of frozen pulverized tumor were homogenized, filtered, and centrifuged. Chicken red blood cells were added as an internal standard, and the cells were lysed and stained for DNA. DNA-stained nuclei were isolated and analyzed using an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Frequency distributions of presynthetic (G_0-G_1), synthetic (S-phase), and postsynthetic/mitotic (G_2-M) cells were evaluated using a modeling program (MODFIT; Verity Software House, Inc., Topsham, ME). G_0-G_1 and G_2-M components were modeled as Gaussian distributions, S-phase components were modeled as single trapezoids, and debris was modeled as a single-cut distribution (10).

Immunostaining and Scoring of Slides. Slides were immunostained using primary antibody 14C10 at 10 μg/ml and a standard streptavidin-biotin detection system. Antigen retrieval was performed with boiling citrate buffer in a pressure cooker for 2 min prior to incubation with primary antibody. The chromogen was $H_2O_2^-$ diaminobenzidine, which was postenhanced with 0.2% osmium tetroxide. Methyl green was used as a counterstain. Nonimmune mouse IgG at 10 μg/ml was substituted for the primary antibody in negative controls. Reactive lymphoid germinal centers and basal crypt epithelium in human appendix were used as positive controls for dividing cells (Fig. 1).

Immunostained slides were evaluated microscopically at ×400 magnification and scored for percentage of positive tumor cells. In larger samples (containing more than about 500 tumor cells), this involved point-counting with a 10 × 10 × 1 mm occular grid, assessing at least 100 tumor cells at intersects, and the fields counted were chosen randomly. In smaller samples, this involved counting all the tumor cells in the specimen.

Statistical Considerations. Relationships between mitosin staining and other prognostic markers were assessed by Spearman rank correlation coefficients. Two clinical outcomes were evaluated: DFS, defined as the time from diagnosis to first recurrence or to last contact, and OS, defined as the time from diagnosis to death (from any cause) or last contact. For survival analyses, clinical variables were dichotomized or trichotomized using previously established cutpoints: age (≤50 *versus* >50 years), tumor size (≤2 *versus* >2 cm), ER (<3 *versus* ≥3 fmol/mg protein), PgR (<5 *versus* ≥5 fmol/mg protein), and S-phase fraction (<6, 6–10, and >10%). Univariate survival curves were estimated using the method of Kaplan and Meier (11) and compared using the log rank test (12). Cutpoint analysis was used to select an optimal cutpoint for mitosin staining. Cox's proportional hazards regression model (13) was used to assess the independent prognostic contribution of clinical variables. For each outcome (DFS and OS), forward stepwise variable selection was used to identify a reduced set of explanatory variables. Final Cox regression models were chosen by forward selection (P to enter, 0.10). Wald χ^2 statistics were used to estimate the significance of individual regression coefficients (13) after adjusting for other factors, and hazard ratios were used to report the prognostic effect size. Optimality of the selected models was verified by all-possible-subsets analyses. Analyses were performed using SAS (version 6.09; SAS Institute, Cary, NC) and S-PLUS (version 3.1; Statistical Science, Seattle, WA).

RESULTS

Distribution of Mitosin Scores and Other Factors. The distribution of clinical, pathological, and biological features (including mito-

Table 1 *Distribution of clinical, pathological, and biological features of the patients and tumors included in this study*

Variable	*n*	Mean	SD	Median	Minimum	Maximum
Mitosin (%)	386	9.1	8.3	7.0	1	47
SPF (%)	294	11.0	8.3	8.7	0	48
Patient age (yr)	386	59.8	13.5	60.4	27	102
Tumor size (cm)	369	2.9	1.5	2.5	0.5	11.0
ER (fmol/mg)	386	105.0	176.5	38.0	0	1711
PgR (fmol/mg)	370	134.7	312.4	10.1	0	3232

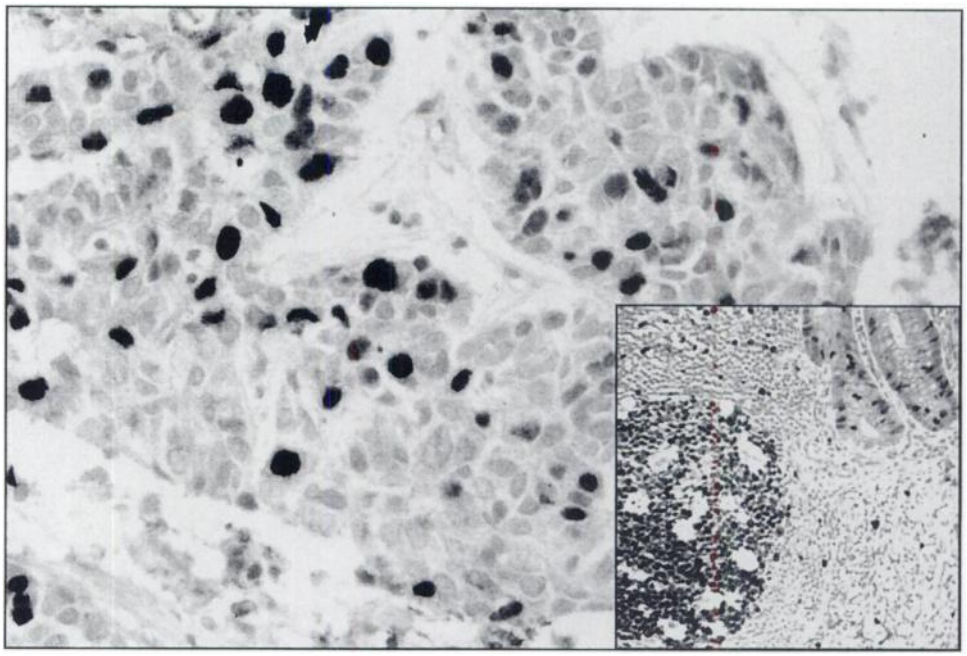

Fig. 2. Representative example of a human breast cancer from this study immunostained for mitosin with antibody 14C10 (×400 magnification). Positive cells show a dark brown nuclear signal. In this example, 27% of the cells are positive or proliferating. *Inset*, human appendix used as a positive control with proliferating cells, largely restricted to lymphoid germinal centers and basal crypt epithelium (×100 magnification).

sin) of the patients and tumors used in this study are summarized in Table 1. The immunostaining signal for mitosin was located in the nucleus of tumor cells (Fig. 2). Positive cells were interpreted as cycling cells. The range of positive cells in the entire study population of 386 tumors was 1–47%, and the median was 7.0%. There was no evidence of degradation of the mitosin signal as a function of the length of time that the tumor samples had been in the freezer (Spearman correlation coefficient = −0.04, $P = 0.46$).

Associations with Clinical/Pathological Features. Spearman rank correlation coefficients between the percentage of mitosin-positive tumor cells and other factors are shown in Table 2. SPF is the most clinically validated method for measuring proliferation in the prognostic evaluation of breast cancer (14, 15), and there was a strong positive correlation between mitosin scores and SPF ($r = 0.57$), both in diploid and aneuploid tumors. There were significant negative associations between mitosin and steroid receptor levels and patient age. No significant relationship was observed with tumor size.

Table 2 *Spearman rank correlations between the percentage of mitosin-positive tumor cells and other clinical, pathological, and biological features of the patients and tumors included in this study*

Variable	Correlation coefficient	P	n
SPF	0.57	0.0001	294
Diploid	0.52	0.0001	101
Aneuploid	0.56	0.0001	193
Tumor size (cm)	0.06	0.23	369
Patient age (yr)	−0.23	0.0001	386
PgR	−0.35	0.0001	370
ER	−0.40	0.0001	386

Associations with Clinical Outcome. Mitosin was first analyzed as a continuous variable in univariate analyses. There were no significant correlations between mitosin and DFS ($P = 0.165$) or OS ($P = 0.980$). Logarithmic transformation improved the model fits but without achieving statistical significance. SPF was analyzed in a similar manner with the same negative results.

We then searched for an optimal cutpoint for mitosin that might dichotomize patients into subsets with significantly different risks, in a manner similar to what we have done for SPF in previous prognostic studies of breast cancer (10). A narrow range of cutpoints (8–11%) was identified that provided statistically significant differences between DFS curves for patients with low and high levels of mitosin-positive cells. A cutpoint of 9% provided the optimal separation ($P = 0.0161$; Table 3). After adjustment for multiple comparisons (16), the difference using the 9% cutpoint was marginally statistically significant ($P_{adj} = 0.10$) and separated patients into low-risk and high-risk subsets, with 84% and 68% DFS at 5 years, respectively (Fig. 3). No significant cutpoints were found for mitosin in predicting OS. In the same group of patients, SPF as a dichotomous variable was also significantly related to DFS but not OS.

A multivariate analysis using the 9% adjusted cutpoint was performed in the subset of patients having complete data for mitosin, ER, PgR, tumor size, and patient age ($n = 353$). Using DFS as the endpoint, tumor size (>2 cm; relative risk = 2.12) and high mitosin (>9% tumor cells; relative risk = 1.62) were significant independent predictors of outcome (Table 3). An analysis was also performed in a smaller subset of patients in which SPF was also available, in addition to all the other variables ($n = 263$). Again, only tumor size (relative

Table 3 *Univariate and multivariate analyses of DFS (353 patients with 92 recurrences) and OS (353 patients and 79 deaths) using mitosin as a dichotomous variable (cutpoint, >9%)*

	Disease-free survival			Overall survival		
Variable	Univariate P	Multivariate P	Relative risk (95% confidence interval)	Univariate P	Multivariate P	Relative risk (95% confidence interval)
Tumor size (cm)	0.0014	0.0022	2.1 (1.3–3.4)	0.0124	0.0139	1.9 (1.1–3.1)
Mitosin	0.0161	0.0222	1.6 (1.1–2.4)	0.8336	0.9276	
ER	0.4947	0.1041		0.5323	0.1545	
Patient age (yr)	0.5690	0.1672		0.2220	0.1591	
PgR	0.9902	0.2152		0.6323	0.8778	

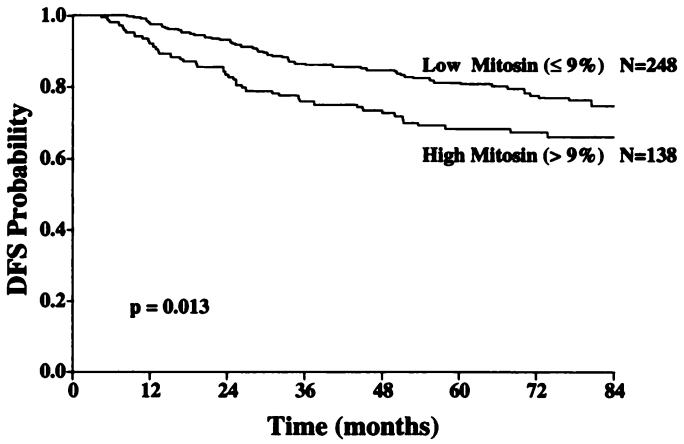

Fig. 3. Kaplan-Meier curves of DFS in 386 node-negative breast cancer patients dichotomized as having low or high rates of mitosin-positive cells (cutpoint = 9%).

risk = 2.47) and mitosin (relative risk = 1.72) were significant independent predictors of DFS, demonstrating that mitosin is superior to SPF as a prognostic factor measuring proliferation in these patients.

To minimize the potentially confounding effects of adjuvant therapy, separate multivariate analyses were performed that excluded patients who received any form of systemic adjuvant therapy, and the results were nearly identical to those above, although the levels of significance for tumor size and mitosin were slightly reduced, due to smaller sample size.

Neither mitosin nor SPF was associated with OS in multivariate analyses in this group of patients, which may be partially due to the small number of deaths in this relatively good-prognosis group of patients.

DISCUSSION

SPF is the most clinically validated method of measuring proliferation in the prognostic evaluation of breast cancer (14, 15). Despite its prognostic usefulness, SPF is technically challenging and expensive, which, among other reasons, has motivated a search for alternative methods for assessing proliferation, such as IHC against cell cycle-related antigens. Relative to SPF, IHC has the advantages of being less expensive, easier to perform, and adaptable to routine formalin-fixed, paraffin-embedded tumors, regardless of size.

Mitosin, because of its direct participation in mitotic-phase progression (1, 2), is an attractive potential target for the quantitative assessment of proliferation by IHC in routine archival tumor tissue. This pilot study is the first to evaluate and demonstrate significant relationships between mitosin expression and SPF, other standard prognostic factors, and clinical outcome in any setting.

In addition to mitosin, there are several other potential antigenic targets for measuring tumor proliferation by IHC, including Ki67 (17, 18) and proliferating cell nuclear antigen (17, 19), among others. Ki67 has been the most thoroughly evaluated antigen, and previous studies have shown only weak positive correlations between Ki67 and SPF (17, 18). In contrast, this study showed a very strong positive correlation between mitosin and SPF. Mitosin was inversely related to ER, PgR, and patient age, which is similar to Ki67 (17, 18) and SPF (4, 8, 9) in previous studies.

Here, both mitosin and SPF were associated with early disease recurrence in univariate analyses when evaluated as dichotomized (mitosin) or trichotomized (SPF) variables. However, in a multivariate analysis, tumor size (relative risk = 2.47) and mitosin (relative risk = 1.72) were the only independent predictors of patient outcome in a model containing several standard prognostic factors, including tumor size, patient age, steroid receptors, and SPF. This suggests that mitosin, as assessed by IHC, has potential as a replacement for SPF as a prognostic factor in breast cancer. In a recent comprehensive study of Ki67 in breast cancer, both SPF and Ki67 provided significant independent prognostic information (18), suggesting that Ki67 may complement rather than replace SPF.

These preliminary results with a first-generation antibody suggest that mitosin may be superior to SPF as a measure of proliferation in the prognostic evaluation of node-negative breast cancer, although this will have to be validated in additional clinical studies. In addition to its potential prognostic superiority, mitosin assessed by IHC is easier to perform and is less costly than SPF, which may facilitate wider use of tumor proliferation as a prognostic factor in breast cancer patients.

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